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13. ABSTRACT *(Maximum 200*

Human breast cancer is characterized by the inappropriate expression of growth factors, kinases and possibly certain transcription factors. Our project focuses on the potential role of a family of transcription factors known as NF-kB/Rel in human breast cancer. Additionally, the project will analyze a role for VNTR elements in the hereditary susceptibility of women to breast cancer and on a role for estrogen in modulating cell growth of human breast cancers. It has been reported previously that certain genes known to be regulated by NF-kB/Rel proteins are inappropriately activated in human breast cancer. We have explored mechanisms that may be involved in the activation of NF-kB/Rel proteins in human breast cancer. Our data rule out the general mechanism of induced nuclear translocation, but new data indicate that NF-kB can be activated through a novel mechanism involving targeting of the transcriptional activation function of the NF-kB p65 subunit. We are presently exploring the role and requirement of NF-kB in human breast cancer and whether this transcription factor is involved in responses to oncogenes, to estrogen and whether it controls gene expression induced by VNTR elements.

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FOREWORD

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Albert S Baldwin 7/23/96

PI - Signature Date

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INTRODUCTION:

Human breast cancer is characterized by altered expression of growth factors, growth factor receptors and kinases (Lippman and Dickson, 1989). Downstream modulators of growth factors and kinases are transcription factors which likely modulate altered responses in breast cancer. Genetic analysis also indicates that other factors are involved in development or progression of human cancer, including repetitive DNA elements called VNTR elements (Krontiris et al, 1993), the recently cloned BRCA1 gene (Futreal et al, 1994), and the tumor suppressor gene p53 (Rotter et al, 1993). Additional emphasis has been placed on the role of estrogen in the development of breast cancer.

NF-kB/Rel proteins are widely distributed and are typically found in the cytoplasm where they are associated with an inhibitor protein called IkB (Beg and Baldwin, 1993). Upon exposure to inflammatory cytokines or growth factors, NF-kB factors dissociate from the IkB inhibitory proteins and translocate into the nucleus (Finco and Baldwin, 1995). In the nucleus, these dimeric factors regulate transcription of genes that contain the kB binding site. Typically these genes encode proteins involved in immune and inflammation responses but more recent work has shown that genes encoding growth regulatory proteins (such as c-myc) are regulated by NF-kB (see Baldwin, 1996).

NF-kB and IkB proteins are associated with oncogenesis. For example, members of the NF-kB family of proteins are related to the product of the c-Rel proto-oncogene which is found overexpressed in certain tumor cell lines. Additionally, the p100 member of the NF-kB family is found translocated in certain lymphomas as is Bcl-3, a member of the IkB family (see Beg and Baldwin, 1993; Baldwin, 1996). Based on our preliminary data, we have proposed that dysregulation of normal NF-kB regulation (i.e., chronic nuclear localization of some forms of NF-kB) may play an important role in the development or progression of human breast cancer. However, new data indicates a second mechanism whereby NF-kB may be activated and separate new data indicates a mechanism whereby NF-kB appears to function in oncogenesis (see Progress/Results section). These new results takes on broadly significant implications regarding oncogenesis in general and particularly in regards to breast cancer since it has been published this year that oncogenic HER2/Neu (an oncogene activated in a significant number of breast cancers) can activate transcription through NF-kB binding sites (Galang et al., 1996).

We have previously found that a breast cancer cell line exhibited constitutive activation of NF-kB. Furthermore, we had found that estrogen treatment of a fibroblast caused enhanced expression of a reporter gene that is controlled by multiple NF-kB sites. Furthermore, some genes shown to be overexpressed in human breast cancer (for example, vimentin and ICAM-1) are known to be regulated by NF-kB. Thus one broad aim of the proposal (Dr. Baldwin's part of the project) was to study the expression of NF-kB/Rel proteins in human breast cancer and to study the functional outcome of this potential overexpression. Our new findings do not alter the aims that were originally planned but they do alter how we approach them (see below). Additionally, these results raise questions regarding whether oncogenes associated with breast cancer (HER2/Neu or TC21) may functionally target NF-kB as part of the transformation process.

A second focus of this proposal is the functional study of human VNTR elements. These DNA elements are repetitive seqences and certain rare alleles of these repeats are associated with an increased risk in the development of breast cancer (Krontiris et al., 1993). These elements arise from the head to tail concatenation of short sequence motifs. The proto-oncogene Ha-ras1 is tightly linked to a VNTR. This VNTR consists of 30-100 copies of a 28 bp DNA element. Krontiris first showed that rare alleles of Ha-ras appear in the genome of cancer patients at a higher frequency that in non-affected women. More recent data indicate that these rare alleles are found more frequently in African-Americans and are correlated with an increase of breast cancer in this population. It has been published that VNTRs bind to NF-kB. The broad aims of this part of the proposal (Dr. Conway) are to explore a role for VNTR elements in human breast cancer, exploring the correlation of rare alleles with breast cancer patients and further characterizing the factors that bind to VNTR elements.

As stated above, rare alleles of the Ha-ras VNTR occur more frequently in individuals with cancer, including breast cancer, than in those without cancer (Garrett et al., 1993; Krontiris, 1994). Although the mechanism of this is association is unknown, one explanation may be that Ha-ras alleles possess a biological function and the rare alleles may function differently than the common alleles. Certain studies indicate that rare Ha-ras alleles bind NF-kB transcription factors more readily, thereby effecting a higher level of transcriptional enhancement than the common alleles (Green et al., 1993); this difference may be dependent upon their respective internal sequences. We have found that Ha-ras VNTR alleles vary not only in repeat copy number but also in the interspersion pattern of repeat sequence variants along the VNTR. Sequence analysis of individual 28 bp repeats shows that a given repeat may possess either a G or a C at positions 7 or 15, giving rise to four possible repeat types. We have a designed a minisatellite variant repeat (MVR)-PCR approach to detect these VNTR sequence variants. Four repeat-specific primers corresponding to the G/C polymorphisms and a common anchored primer outside of the VNTR are used to PCR amplify fragments whose lengths define the positions of the polymorphisms in the repeat unit. In effect, an allele-specific sequence polymorphism ladder is generated. Using this method, we have recently shown that VNTR allele length is very tightly linked to MVR internal sequence (Conway et al., 1996). That is, nearly all alleles having the same length also have the same MVR allelotype. In contrast to the common alleles that are detected repeatedly in a population, rare alleles possess unique and disordered sequences (Conway et al., 1996). Most rare VNTR alleles begin at the 5' end as one common allele then abruptly switch to either a second recognizable motif or become completely unrecognizable. This suggests that rare MVR alleles arise via recombination involving segments of one or more of the common alleles.

The G/C polymorphisms within the 28 bp repeat subunit could potentially affect transcription factor binding, perhaps by influencing methylation patterns or DNA secondary structure. If we are able to more clearly define the differences between rare and common alleles based upon a combination of transcription factor binding, transcriptional enhancement activity, and structural characteristics such as VNTR length and internal sequence variations, we can identify true rare alleles which predispose to breast cancer. To this end, we proposed to investigate the potential regulatory role of the Ha-ras VNTR by characterizing its interaction with members of the NF-kB/Rel family and other transcription factors. This was to be accomplished by evaluating protein binding to 28 bp VNTR subunits carrying specific G/C polymorphisms, and to longer tandem arrays of subunits generated by MVR from either common or rare alleles. We were also interested in characterizing the binding proteins present in a series of nuclear extracts from cell lines and from breast tumor tissues as well as normal mammary epithelium. Finally, we wanted to determine the biological outcome of VNTR/protein interactions by characterizing transcriptional regulatory activity of common versus rare VNTR sequences. Listed below are the original aims of the grant application:

Aim 1 is to investigate the potential biological function of the Ha-ras VNTR through characterization the nuclear factors that bind to this element with a definite focus on the potential interaction of NF-kB/Rel proteins. Further approaches include studies aimed at addressing potential transcriptional activation properties of the VNTR.

Aim 2 is designed to determine if NF-kB/Rel binding to VNTR elements may be used as a more refined method of identifying patients at risk for breast cancer.

Aim 3 is to analyze relative nuclear and cytoplasmic levels of NF-kB/Rel proteins in normal breast epithelium and in human breast cancer. We will correlate NF-kB expression with activation of certain kinases thought to regulate NF-kB expression and with the status of transcriptional activators shown to regulate NF-kB gene expression.

Aim 4 is to correlate expression of NF-kB with expression of known or suspected prognostic markers for human breast cancer (ICAM-1, urokinase and vimentin). We will determine if the ligand for HER2/Neu, NDF, can induce the expression of NF-kB.

Aim 5 is to determine whether estrogen can regulate gene expression through a kB site and whether this is due to the activation of NF-kB/Rel binding activity.

BODY:

Conway Laboratory: Progress and Results

Task 1.

a. Analysis of Protein Interactions with the Ha-ras VNTR

Previous DNA Binding Studies/NF-kB and AP-1 We have been characterizing the potential interaction of the Ha-ras VNTR with transcription factors including those of the NF-kB/rel family using gel mobility shift assays. We have synthesized double-stranded 28 bp oligonucleotides corresponding to each of the 4 Ha-ras VNTR subunit repeat types carrying specific G/C polymorphisms. Oligonucleotides (BstN-1, BstN-2, BstN-3 or BstN-4) were ^{32}P-end-labeled and were incubated with nuclear protein extracts, then were run in non-denaturing polyacrylamide gels to determine which repeat type was bound most tightly by nuclear proteins. Conditions for these experiments were established using nuclear extracts from Jurkat T cell leukemia cells either untreated (uninduced) or following induction with a combination of phytohemagglutinin (PHA) and phorbol myristate acetate (PMA). Gel shifts using either the BstN-4 or BstN-1 28 bp VNTR probes indicated that these sequences exhibited protein binding profiles that appeared to correspond to NF-kB/rel proteins. Two bands of similar size were visible and protein binding to the VNTR sequence was the same for both the induced and the uninduced extracts, indicating that only the constitutive form of NF-kB (p50) appears to bind this sequence.

In an attempt to demonstrate the specificity of protein binding to the Ha-ras VNTR sequence, we used an AP-1 consensus sequence as unlabelled competitor. The transcriptional regulatory factors of the fos and jun families bind to AP-1 sites. The AP-1 sequence competed away protein binding primarily in the lower band but not of the upper band. Alternately, when the NF-kB consensus sequence, UV-21, was used as competitor, the upper band of protein binding disappeared suggesting that the Ha-ras VNTR contains an AP-1 as well as an NF-kB site. In addition, the fact that the UV-21 (NF-kB) and AP-1 competitors reduced binding in different bands suggested that the binding sites may be separated along the length of the repeat. Because proteins that might normally bind AP-1 sites may also bind the Ha-ras VNTR, we carried out additional gel shifts to evaluate the binding of proteins directly to AP-1. Unlike the Ha-ras VNTR sequence, AP-1 binds both constitutively expressed (uninduced) proteins and proteins induced following treatment with PHA and PMA. The UV-21 sequence efficiently competes for binding in the lower constitutive protein band, but not in the upper induced band. The AP-1 and the UV-21 competitors both eliminated binding to the BstN-4 VNTR sequence.

NF-kB Protein Supershift Studies In an effort to identify the putative NF-kB species which bind the VNTR, we have performed preliminary gel supershifts using antisera specific for the p50 subunit of NF-kB (the constitutive form). Briefly, antisera is pre-incubated with the nuclear extract to allow for antibody-protein binding. The antibody-protein mixture is then incubated with the labeled DNA, and these are electrophoresed in non-denaturing polyacrylamide gels. DNA-protein complexes which bind antibody will be seen to migrate more slowly and thus are shifted toward the top of the gel. The results of p50 supershifts have so far been negative suggesting that p50 probably does not bind the VNTR, at least under the conditions used in these experiments. We are in the process of carrying out supershifts with antibodies to other members of the NF-kB family in order to obtain a more definitive answer concerning the exact proteins which might bind the VNTR.

p53 DNA Binding Studies We are also evaluating the potential interaction of the p53 tumor suppressor protein with the Ha-ras VNTR because: (1) the p53 protein binds DNA in a sequence-specific manner and regulates transcription of a variety of genes (Funk et al, 1992; Friend, 1994), (2) careful examination of the Ha-ras VNTR sequence indicates that the types 3 and 4 28 bp subunits (BstN-3 and 4) each possess a perfect p53 half-site and this potential

binding site lies at the G/C polymorphism at position 15, suggesting that p53 could bind the VNTR (see Table 1), (3) p53 is mutated or overexpressed in many breast tumors (Seth et al, 1990; Bartek et al, 1990), (4) our NF-kB p50 supershift studies appear to be negative, indicating that some protein other than p50 interacts with the VNTR, and (5) the p53 protein is similar in size to the NF-kB p50 subunit and would be expected to give similar migration patterns in gel shifts assays.

Table 1: Potential Transcription Factor Binding Sites in the Ha-ras VNTR 28 bp Subunits

A. p53 consensus DNA binding sequence showing the two half-sites (sequence 2 is found in the Ha-ras VNTR)(Funk et al, 1992):

```
3'-CCTGTACGGGCCCGTACAGG-5'
5'-GGACATGCCCGGGCATGTCC-3'
      ↑1        ↑2
```

B. NF-kB consensus DNA binding sequence (sequence 3 is found in the Ha-ras VNTR)(Trepicchio and Krontiris, 1992):

```
5'-GGGGACTTTCC-3'
    ↑3
```

C. Ha-ras VNTR 28 bp repeats showing potential p53 and NF-kB binding sites:

```
                          ←----------28 bp repeat--------→
Type 1 repeat:    3'-CCCCTGCGG...TGTGAGCGGGAAGAGAGGTCCCCTGCGG...TGTGAG-5'
(VNTR consensus)  5'-GGGGACGCC...ACACTCGCCCTTCTCTCCAGGGGACGCC...ACACTC-3'
                                  •       •
Type 2 repeat:    3'-CCCCTGCGG...TGTGAGGGGGAAGAGAGGTCCCCTGCGG...TGTGAG-5'
                  5'-GGGGACGCC...ACACTCCCCCTTCTCTCCAGGGGACGCC...ACACTC-3'
                                  •       •
Type 3 repeat:    3'-CCCCTGCGG...TGTGAGGGGGAAGACAGGTCCCCTGCGG...TGTGAG-5'
                  5'-GGGGACGCC...ACACTCCCCCTTCTGTCCAGGGGACGCC...ACACTC-3'
                                  •       •
Type 4 repeat:    3'-CCCCTGCGG...TGTGAGCGGGAAGACAGGTCCCCTGCGG...TGTGAG-5'
                  5'-GGGGACGCC...ACACTCGCCCTTCTGTCCAGGGGACGCC...ACACTC-3'
                                  •       •2      3
                                  ↑       ↑
                                 G/C     G/C
                                pos.7   pos.15
```

A sequence tract similar to p53 sequence 1 is also found in the Ha-ras VNTR encompassing the polymorphic site at position 7; the degree of sequence similarity is dependent on the repeat type and specific polymorphism.

We have carried out gel shift experiments to evaluate the possible binding of p53 protein to the Ha-ras VNTR. For these studies, we have used nuclear extracts from the Jurkat cell line or the breast tumor lines MCF-7 (wild-type p53) and T47D (mutant p53), and oligonucleotide probes corresponding to VNTR repeat type 1 (low p53 binding potential?) or type 4 (high p53 binding potential?). We have also used the p53 consensus DNA binding sequence (shown in Table 1A) as a positive control in order to determine the pattern of protein binding expected with p53 for comparison with that obtained with the VNTR repeats. The p53 and Ha-ras VNTR

probes exhibited the exact same two bands of protein binding (one major and one minor), regardless of the protein extract used. These bands appeared to be the same as those seen in previous DNA binding experiments using the VNTR, AP-1 and NF-kB (UV-21) probes. There was little effect of induction by PMA and PHA on the quantity of protein bound to the p53 or the VNTR sequences.

Supershift studies to demonstrate that the p53 protein binds the VNTR repeats have been unsuccessful and problematic. Other investigators have reported difficulty in super-shifting p53/DNA complexes (among them, Dr. Baldwin's lab), and interpretation of this type of experiment is complicated by the variety of antibodies available for p53 and the fact that certain antibodies can actually induce stronger complexation of the p53 protein with the DNA target sequence.

Our cumulative results from the gel shift experiments with the Ha-ras VNTR repeat sequences suggest that several proteins may potentially bind the VNTR, and this binding appears to be at least somewhat specific since certain unlabelled competitor sequences can compete for binding while others do not. Those consensus sequences which can compete for binding, in either of the two bands normally seen with the VNTR, are NF-kB, AP-1 and p53. This would suggest that proteins which normally bind these sequences may also interact with the VNTR. However, the limited super-shift assays have thus far failed to identify specific proteins which bind the VNTR. Because of the difficulty in interpreting these data, we are concentrating our efforts on evaluating the transcriptional regulatory effects of the Ha-ras VNTR. Our preliminary work in this area is discussed below. We expect that this line of experimentation will yield more definitive results on the transcriptional pathways which involve the VNTR region. We will then try to more specifically identify the proteins which interact with the VNTR when we have a better indication of which family of transcription factors is most relevant to the issue of transcriptional regulation via the VNTR.

b. <u>Transcriptional Regulatory Function of the Ha-ras VNTR</u>

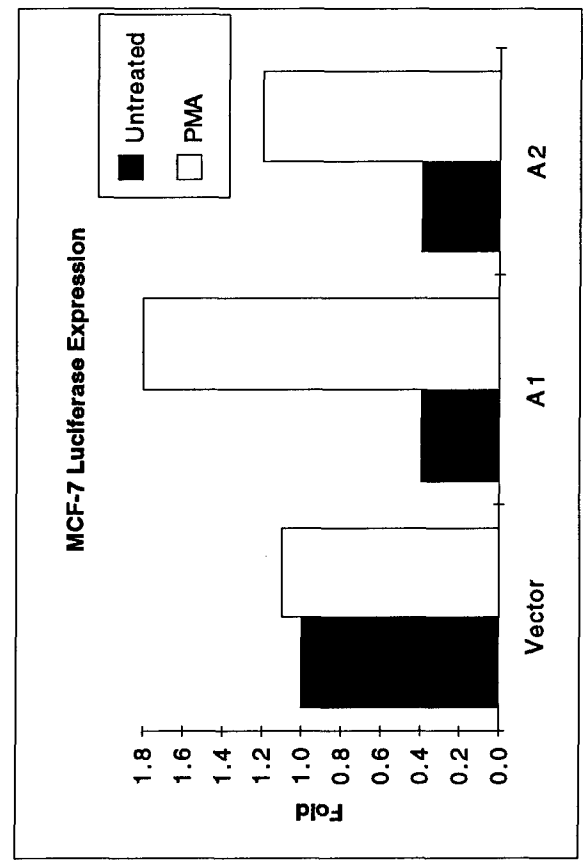
Cloning of Ha-ras VNTR Alleles In order to evaluate potential differential transcriptional regulatory functions of the rare versus common Ha-ras VNTR alleles, we have TA-cloned a large number of Ha-ras VNTR PCR products into TA vectors for use as gel molecular size standards. Several of these VNTR inserts have subsequently been subcloned into luciferase reporters downstream of both the SV-40 promoter and luciferase gene, which is the normal position of the Ha-ras VNTR with respect to the c-Ha-ras gene. The VNTR alleles TA-cloned are listed in Table 2; alleles sub-cloned for luciferase assays are noted.

In the luciferase expression assays, we will compare the transcriptional activity obtained from rare versus common VNTR alleles, or from the shorter common alleles containing 3 (28 bp) repeat types (a1 and a2) versus the longer common alleles (a3 and a4) containing an additional type 4 repeat (a3 and a4). To date, we have sub-cloned the a1 and a2 common alleles and have begun to evaluate their transcriptional regulatory effects. Cloning of the a3 and a4 alleles along with several rare alleles is in progress, however, obtaining full-length clones for these larger alleles has been more difficult due to the smaller quantity of PCR products obtained and the apparent instability of the longer VNTR segments in the bacterial hosts. When comparing rare and common alleles, we have chosen to match common alleles with rare alleles possessing abnormal MVR sequences but common allele lengths. This is so that we can control for potential length effects of the different alleles.

We have classified the VNTR alleles in Table 2 as common, variant or rare, based upon a number of factors including length and MVR sequence. The common alleles shown above include the a1, a2, a3 and a4 alleles that have stable lengths and MVR sequences. Alleles are considered variant if they deviate from a common allele sequence by one or two repeats within the first 20 repeats from the 5' end of the VNTR, even if the length is the same as that of a common allele. Rare alleles have abnormal length and/or sequence and the sequence alteration deviated by more than 2 repeats as compared with the progenitor common allele. Although this has been our operational definition of rare alleles, most alleles we consider to be rare actually have substantially disorganized or rearranged sequences.

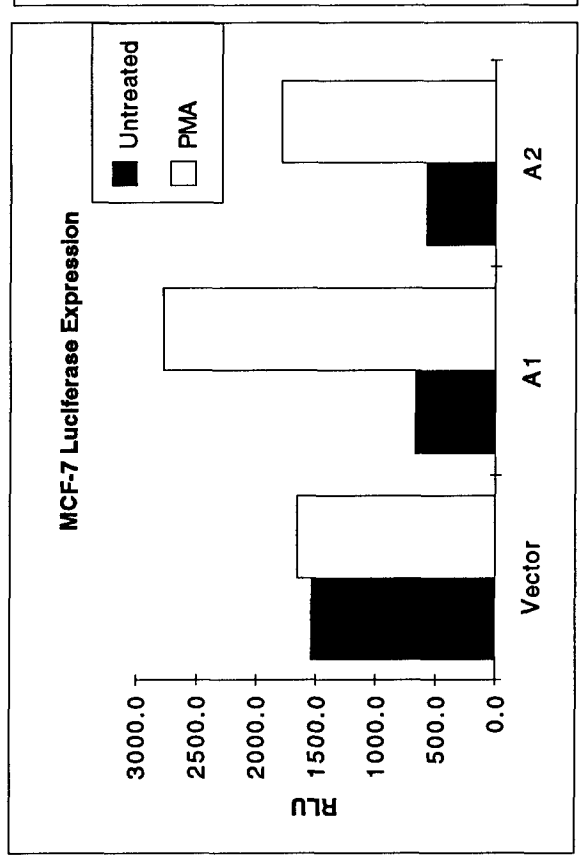
Figure 1

A:


MCF-7 Luciferase Expression
Untreated
PMA
RLU
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Vector
A1
A2

MCF-7 Luciferase Expression
Untreated
PMA
Fold
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Vector
A1
A2

B:


T47D Luciferase Expression
Untreated
PMA
RLU
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900000.0
800000.0
700000.0
600000.0
500000.0
400000.0
300000.0
200000.0
100000.0
0.0
Vector
A2

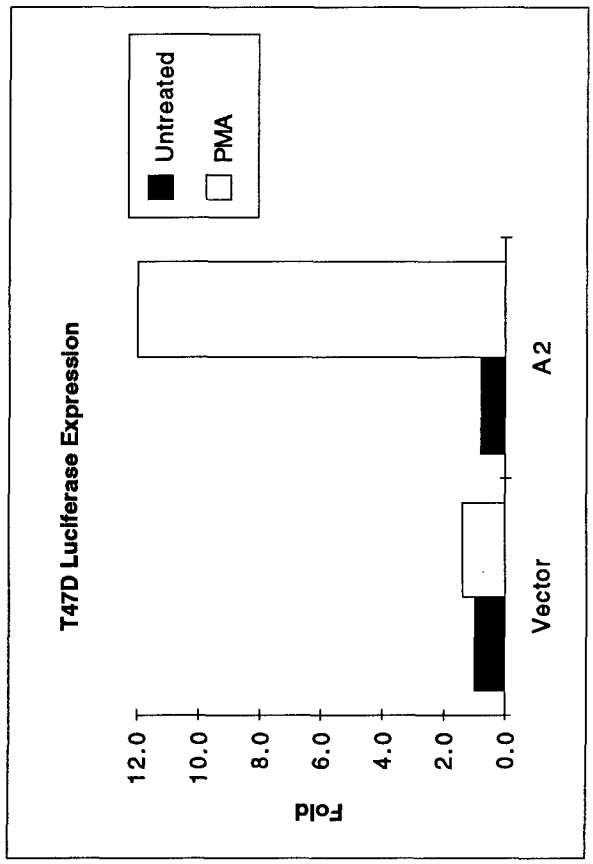
T47D Luciferase Expression
Untreated
PMA
Fold
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Vector
A2

Table 2: Ha-ras VNTR Alleles Cloned for Gel Markers and/or for Luciferase Transcription Assays

Allele	Number of 28 bp Repeats	Allele Status	Number Alleles of this Size Cloned
a1-2	28	common	1
a1-1	29	variant	1
a1	30*	common	2
a1+1	31	variant	1
a1+2	32	common	1
a1+4	34	common	2
a1+6	36	common	2
a1+8	38	rare	1
a1+9	39	rare	1
a1+11	41	rare	3
a1+13	43	rare	2
a1+15	45	variant	3
a2	46*	common	3
a2+1	47	variant	2
a2+9	55	rare	1
a2+13	59	rare	5
a2+15	61	rare	1
a2+19	65	rare	1
a2+20	66	rare	2
a3	69	common	2
a3+1	70	variant	2
a3+3	72	rare	1
a3+4	73	rare	1
a3+9	78	rare	1
a3+12	81	rare	1
a4 group	85	common	1
a4 group	86	common	1
a4 group	87	common	1
a4 group	88	common	1

*Alleles sub-cloned for luciferase expression assays. In instances in which more than one rare allele of a given length was cloned, each of these rare alleles possesses a different MVR sequence.

Transcriptional Regulation by the Ha-ras VNTR Transcriptional enhancement by the Ha-ras VNTR was evaluated in the MCF-7 breast tumor cell line which expresses a low level of wild-type p53. Transient transfections were performed as follows. Cells were plated and allowed to grow to 40-60% confluence, then were transfected with the construct of interest using Lipofectin reagent. Cultures were allowed to grow overnight and the following morning, the medium was changed. At the end of the day, cells either remain untreated or were treated overnight with PMA. The next morning, media was changed again and the cells were harvested by lysis in 0.2 M Tris, pH 7.5 and three cycles of freezing and thawing. Cell extracts containing 10-100 ug of protein were placed in a luminometer, the substrates ATP and luciferin were simultaneously injected and light emission was measured.

As shown in Figure 1A, transfection of either untreated or induced MCF-7 cells with luciferase vector alone produced a similar low background level of transcription. Luciferase

expression is given in fold expression or relative loght units (RLU). Transfection with the a1 or a2 common VNTR alleles downstream from the luciferase gene suppresses transcription by about 50-60% in uninduced cells, but stimulated transcription in the PMA-induced cells. The transcriptional enhancement observed was 1.8-fold for a1 or 1.2-fold for a2.

Transfection of the T47D breast tumor cell line, which possesses a much higher level of mutant p53 protein, with vector alone produced a higher basal level of transcription than that seen with MCF-7. When the a2 allele was transfected into T47D, transcriptional repression was not observed in uninduced cells, but the level of transcriptional enhancement was about 12-fold in the induced cells.

These results are preliminary and further experiments are in progress to dissect out the regulatory pathway operating via the Ha-ras VNTR. However, our results are extremely encouraging and indicate that the VNTR does indeed appear to possess transcriptional regulatory function, although it remains unclear which proteins interact with this region and modulate transcription. Interestingly, the MCF-7 and T47D cell lines both express high nuclear levels of NF-kB proteins so that a factor other than NF-kB may have led to the differential transcriptional activity between these two cell lines.

Our data also raise the possibility that p53 status of cells (wild-type or mutant) influences the transcriptional regulatory effects mediated by the VNTR. We plan to continue transfections using the K562 or SAOS-2 cell lines which are deficient in p53 protein. We would expect that if the VNTR enhances transcription via a p53 pathway, transfection of the VNTR reporter into a p53-deficient cell line would fail to enhance transcription. Subsequent co-transfection of the VNTR reporter with a p53 wild-type or mutant expression vector should then restore transcriptional potential (either for repression or enhancement).

We will perform similar co-transfections with the VNTR-reporter and plasmids expressing the p50, p65, or rel proteins into cell lines that exhibit low basal levels of NF-kB. CAT constructs will also be transfected into breast tumor cell lines BT-20 and T47D which express high nuclear levels of NF-B proteins.

Ultimately, our most important goal is to determine whether the rare and common alleles are functionally different. We are continuing to sub-clone the a3 and a4 common alleles and a series of rare alleles, and expect that in the near future we can test this hypothesis.

Immunohistochemical Staining for c-Ha-ras Overexpression in Breast Tumors The Ha-ras protooncogene itself is the most likely target for transcriptional regulation by the VNTR and is certainly important in cellular growth and differentiation. In fact, the tyrosine kinase receptor Her-2/neu, which is amplified and overexpressed in many breast tumors, transduces its cellular signal via ras pathways. One possible consequence of transcriptional enhancement by rare Ha-ras alleles may be the overexpression of the Ha-ras protooncogene upstream of the VNTR. Overexpression of p21 ras protein has previously been observed in the absence of mutation in breast tumors using a pan-p21 ras antibody (Ohuchi et al, 1986). In order to determine whether c-Ha-ras overexpression may be associated with the presence of rare VNTR alleles in breast tumors, we are measuring the expression of this protein by immunohistochemistry in breast tumors using the Ab-1 antisera specific for c-Ha-ras (Oncogene Science). The appropriate conditions are currently being established for staining with this antisera in formalin-fixed breast tumor tissue sections obtained from the Lineberger Comprehensive Cancer Center Tissue Procurement and Analysis Facility. DNA will be isolated from adjacent frozen tissues sections from these same tumors or from corresponding germline DNA for Ha-ras VNTR allelotyping (length and MVR-sequence analyses). We can then determine if VNTR allele status correlates with the level of c-Ha-ras protein expression.

CONCLUSIONS

Conway Laboratory:

(1) Protein Binding to the Ha-ras VNTR Our gel-shift studies suggest that several proteins may bind the Ha-ras VNTR, although our experiments to identify these proteins are still inconclusive. Potential VNTR-binding proteins include certain NF-kB family proteins, AP-1 proteins and p53. These conclusions are extremely tentative and are based solely upon the competition for protein binding by the NF-kB, AP-1 and p53 consensus oligonucleotides in the gel mobility shift experiments. The possibility that members of all three of these transcription factor families might bind the VNTR is unusual and might indicate that either these proteins complex with another protein which directly binds the VNTR, or they each interact with different sequences along the VNTR. We plan to continue supershift experiments using antibodies specific for other members of the NF-kB protein family not yet tested (p52, p65, p49 and c-rel), AP-1 binding proteins (c-fos, c-jun and fra-1) and the ets oncogene protein. However, our highest priority is to continue the transcriptional regulatory studies. We expect these to be more productive and may provide direct evidence for the involvement of the Ha-ras VNTR with a specific transcriptional regulatory pathway.

(2) Transcriptional Effects of the Ha-ras VNTR

Our transient transfection experiments support the idea that the Ha-ras VNTR does indeed possess a transcriptional regulatory function. Depending upon the cell line transfected and the presence or absence of induction by PMA, the VNTR induced either transcriptional repression or enhancement. Although further experiments are needed, p53 protein status (wild-type or mutant) may also affect VNTR regulatory activity. These results are potentially very important because if the VNTR possesses a biological function, it may be a direct participant in the carcinogenic process instead of simply a marker of genomic instability due to another separate locus. The fact that p53 mutation may influence VNTR regulatory activity suggests that Ha-ras VNTR allelotyping (rare or common) might be best evaluated in the context of p53 status.

Publications Resulting from this Work

Conway, K., Edmiston, S.N., Hulka, B.S., Garrett, P.A., Liu, E.T. Internal sequence variations in the Ha-ras VNTR rare and common alleles identified by minisatellite variant repeat (MVR)-polymerase chain reaction (PCR). Cancer Res., In press (1996).

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BODY (Baldwin Laboratory):

Progress/Results:.

Immunohistochemical analysis of NF-kB p65 expression in human breast cancer. We have largely accomplished one major aspect of Aim 3; that is, analyzing the expression of NF-kB/Rel proteins by immunohistochemical studies of human breast cancer sections. Utilizing the antibody against the human NF-kB p65 subunit, it was found that approximately 30-35% of human breast tumors express extraordinarily high levels of NF-kB, as compared to normal breast epithelium. Interestingly, the majority of these exhibit cytoplasmic levels of p65, although NF-kB is nuclear in several examples. We have analyzed expression of other NF-kB forms including the p50 subunit and c-Rel and find no increase in these cells. Additionally, levels of IkBa and of the IkB-like protein Bcl-3 were found to be unelevated in these tissue samples as compared to normal breast epithelium.

NF-kB functional activity can be regulated without induced nuclear translocation. As described above, we have been unable to show that a significant number of breast cancer tumors or breast cancer cell lines exhibit elevated levels of NF-kB, yet breast cancers have elevated transcripts of genes known to be regulated by NF-kB. Thus, we have explored a new avenue: determining if NF-kB dependent gene expression can be up-regulated without enhanced nuclear translocation of this transcription factor. We have initiated our studies using the oncogene Ras and its downstream effector Raf. We chose this model simply because oncogenic Ras and Raf expressing cell lines were available to us and because the signaling pathways directed by these oncogenes are well studied. We show (see Fig. 1 in the Appendix) that oncogenic Ras or oncogenic Raf potently activates gene expression through an NF-kB binding site. However, oncogenic Ras or Raf (Fig. 2) do not increase nuclear translocation of NF-kB. This presented an enigma to us: how is it that NF-kB functional activity can be increased without an increase in nuclear NF-kB. So we tested whether the innate transcriptional activity of the NF-kB p65 is increased under these conditions. Our experiments indicate that the transcriptional activation domain of the p65 subunit of NF-kB is functionally targeted by oncogenic Ras or oncogenic Raf (Fig. 3). Thus, these data indicate that two oncogenes (Ras or Raf) can potently stimulate transcription of NF-kB dependent transcription without inducing nuclear translocation of NF-kB. This is accomplished apparently by the targeting of the transcriptional function of the p65 subunit which exists at low, consitutitive levels in most cells.

NF-kB is required for several oncogenes to neoplastically transform cells. With the above data in mind, we have asked whether NF-kB is required for oncogenic Ras to transform cells. We now show that the ability of Ras to transform cells is blocked by expression of the NF-kB inhibitor IkBa (Fig. 4). Additionally, we show that cells that have the NF-kB p65 genes deleted are incapable of transformation by the oncogene Raf (Fig. 5). Thus, these data indicate that the NF-kB subunit p65 is required for at least two oncogenes to transform cells. We are obviously very interested in determining if transformation by the breast cancer oncogene HER2/Neu requires NF-kB (see goals for the upcoming year).

NF-kB functional activity blocks apoptosis. Protection from apoptosis is a critical component in neoplastic transformation and in protection from radiological and chemotherapies (Fisher, 1994).. Our data now indicate that NF-kB activation protects cells from killing by several cancer therapies. Data shown in Fig. 6 indicates that NF-kB protects cells against killing by tumor necrosis factor. The experimental approach was to express a super-repressor form of IkBa (mutated in serines 32 and 36, which results in a protein that can bind to NF-kB but which cannot be degraded) in the HT1080 fibrosarcoma cell line. This cell line was chosen since it is highly resistant to killing by TNF. Expression of IkBa blocks NF-kB nuclear translocation in these cells and correspondingly sensitizes to killing by TNF. Further evidence (not shown) that NF-kB is protective against killing by TNF are the following observations: (1) re-expression of

NF-kB subunits in the cells expressing IkBa restores protection against killing, (2) fibroblasts that are null for the p65 NF-kB subunit (from the p65 knockout) show enhanced killing by TNF, and (3) cells that are highly sensitive to killing by TNF do not activate NF-kB in response to this cytokine. Thus, these data strongly indicate that NF-kB protects cells against killing by TNF.

Since several cancer chemotherapies kill transformed cells by the induction of apoptosis (Fisher, 1994), we have determined if NF-kB protects against killing by these treatments. We have focused on 2 standard cancer therapies: ionizing radiation and daunorubicin. Our data (not shown, but very similar to that presented in Fig. 6) indicate that the inhibition of NF-kB potently enhances cell killing in response to radiation and daunorubicin treatment. Interestingly, radiation and daunorubicin activate NF-kB but adriamycin does not. Thus, our data indicate that cancer therapies that kill and activate NF-kB are (at least partially) protected against cell killing. Thus, we propose that inhibitors of NF-kB will potently enhance cell killing by ionizing radiation and daunorubicin (and potentially other therapies).

Negative finding: NF-kB does not appear to be activated via nuclear translocation in a significantly large number of human breast cancer tumors. However, new insight into the problem is derived from our studies.

Positive findings: As explained above, we have made a significant breakthrough in understanding mechanisms of oncogenesis that involve and require NF-kB. Briefly, our data indicate that NF-kB is required for many or all oncogenes to transform cells. Additionally, our data indicate that the activation of NF-kB provides protection against apoptotic cell killing, whether spontaneous or induced by cancer radiological or chemotherapies. These findings allow for new approaches in establishing a role for NF-kB proteins in controlling breast cancer.

MORE EXPLICIT EXPERIMENTAL DETAILS ARE DESCRIBED IN THE FIGURE LEGENDS TO THE FIGURES PROVIDED IN THE APPENDIX.

Goals for the upcoming year (Baldwin laboratory): (1) Relative to the original aim 3, we will explore whether the "new" pathway for the activation of NF-kB function is operative in breast cancer cell lines; (2) relative to the original aim 4, we will explore whether Her2/neu activates kB-dependent transcription (as recently reported) through the targeting of the transcriptional activation domain of p65; and (3) relative to the original aim 5, we will determine whether estrogen affects the ability of the p65 transcriptional activation domain to be functional.

CONCLUSIONS (BALDWIN LABORATORY)

First, our data indicate that gene expression directed through NF-kB binding sites can be controlled by a previously unknown mechanism (i.e., direct targeting of low levels of constitutively active [nuclear] NF-kB) without induced nuclear translocation. This is a fundamentally important observation with wide implications. Second, our data indicate that NF-kB is required for certain (possibly all) oncogenes to neoplastically transform cells. This is a broadly significant conclusion since the inhibition of NF-kB by known inhibitors may prove useful in cancer therapy. Third, we find that NF-kB activity protects cells against apoptosis. This particular finding may explain the requirement for NF-kB in transformation since protection against apoptosis is likely to be a critical component of oncogenesis. Each of these finding is fundamentally important to our understanding of cancer, and are likely to be essential for the development and progression of breast cancer. These data provide an important new foundation for pursuing our original aims and provide hope for developing a new generation of cancer therapies based on the inhibition of NF-kB.

MANUSCRIPTS IN PREPARATION:

C.-Y. Wang, M. Mayo, and A. Baldwin. NF-kB is a negative regulator of apoptosis induced by TNF, radiation and daunorubicin. Manuscript in preparation, planned submission to Science.

T. Finco, J. Westwick, A. Beg, D. Baltimore, C. Der and A. Baldwin. NF-kB is activated by and required for transformation by several oncogenes. Manuscript in preparation, planned submission to Cell.

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APPENDIX (BALDWIN LABORATORY)

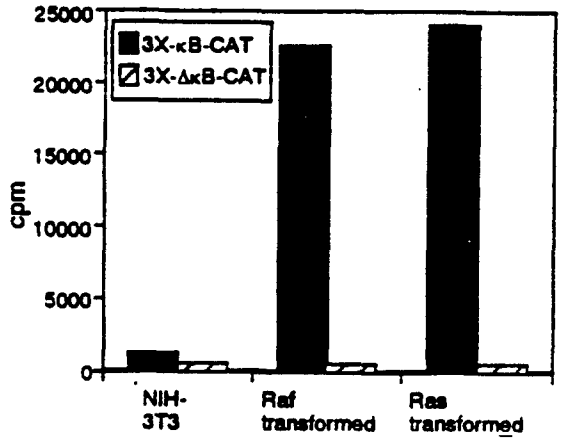

Figure 1. Oncogenic Ras or Raf activate gene expression through NF-kB binding sites. Normal NIH 3T3 cells or those transformed with oncogenic Ras or Raf (gift of Dr. C. Der, UNC) were transfected with a chloramphenicol acetyltransferase reporter (CAT) linked to a minimal fos promoter that contains either 3 wild-type (3X-kB-CAT) or mutated NF-kB sites (3X-deltakB-CAT). Extracts were prepared and CAT assays performed (the solid bars indicate the response to the wild-type reporter and the hatched bars indicate the response to the mutated reporter). The data are reported as cpm incorporated into chloramphenciol and is the average of 5 experiments. The data clearly indicate that oncogenic Ras or oncogenic Raf lead to the potent up-regulation of gene expression directed through an NF-kB site.

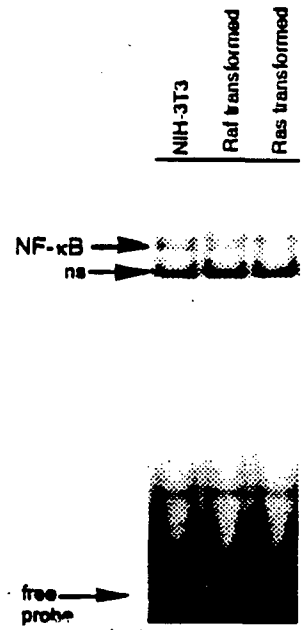

Figure 2. Ras or Raf transformed cells do not exhibit accumulation of nuclear NF-kB. Nuclear extracts from the cells described in Figure 1 were utilized in a gel mobility shift assay using a 32P-labeled NF-kB binding site DNA probe. The nuclear extracts are prepared from sucrose pad purified nuclei from parental 3T3, Ras or Raf transformed cells. The DNA/protein complexes are electrophoresed on non-denaturing 5% polyacrylamide gels. The dried gel is exposed for autoradiography. NF-kB is shown by an arrow and a non-specific band is marked with ns. The free probe is also shown. The data show that nuclear NF-kB does not accumulate in Ras or Raf transformed cells.

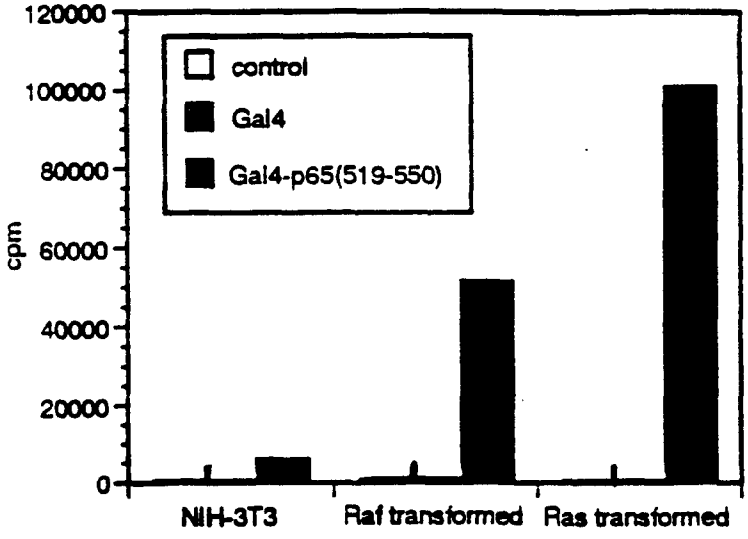

Figure 3. The transcriptional activation domain of p65 NF-kB subunit is activated in Ras or Raf transformed cells. In order to be able to understand why kB-dependent transcription is elevated in Ras or Raf transformed cells in the absence of an increase of nuclear NF-kB, we have performed transient transfection experiments (as described in Fig. 1) utilizing 2 plasmids: one is a CAT reporter directed by binding sites for the GAL4 DNA binding domain and the second is either a plasmid encoding a fusion between GAL4 DNA binding domain and the transcriptional activation domain of p65 or just the GAL4 DNA binding domain alone. The data is presented as cpm incorporated into chloramphenicol and is an average of 4 experiments. The data clearly indicate that the transcriptional activation domain of the p65 NF-kB subunit is activated in Ras or Raf transformed cells.

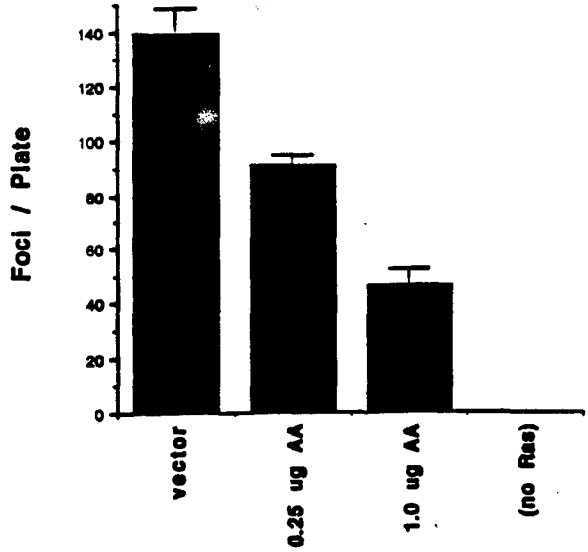

Figure 4. Expression of a super-repressor form of IkBa blocks the ability of oncogenic Ras to transform 3T3 cells. Ras was either transfected into NIH 3T3 cells either with a plasmid encoding a form of IkBa that is mutated in the serines 32 and 36 (this protein functions as a super-repressor by binding NF-kB but is incapable of degradation, thus it blocks nuclear translocation of NF-kB) or with the empty vector. AA denotes the super-repressor mutant. Transformed cell foci were counted; the data is presented as foci per plate. These results have been repeated at least 3 times with similar numbers. The data strongly indicate that NF-kB is required for oncogenic Ras to transform cells.

	No Raf	Raf (p65 +/+)	Raf (p65 -/-)
Foci Per Plate	1	14	1

Figure 5. p65 null fibroblasts are incapable of transformation by oncogenic Raf. We have performed one experiment (which clearly is preliminary). However the data indicate that oncogenic Raf is unable to transform the p65 null fibroblasts, while it can transform wild-type fibroblasts. The data show the number of foci on the control plate without transfection of 3T3 cells with Raf (No Raf), with transfection of oncogenic Raf into wild-type cells (Raf p65 +/+), or with transfection of oncogenic Raf into p65 null cells (Raf p65 -/-). The data indicate that the p65 null fibroblasts are incapable of transformation by oncogenic Raf.

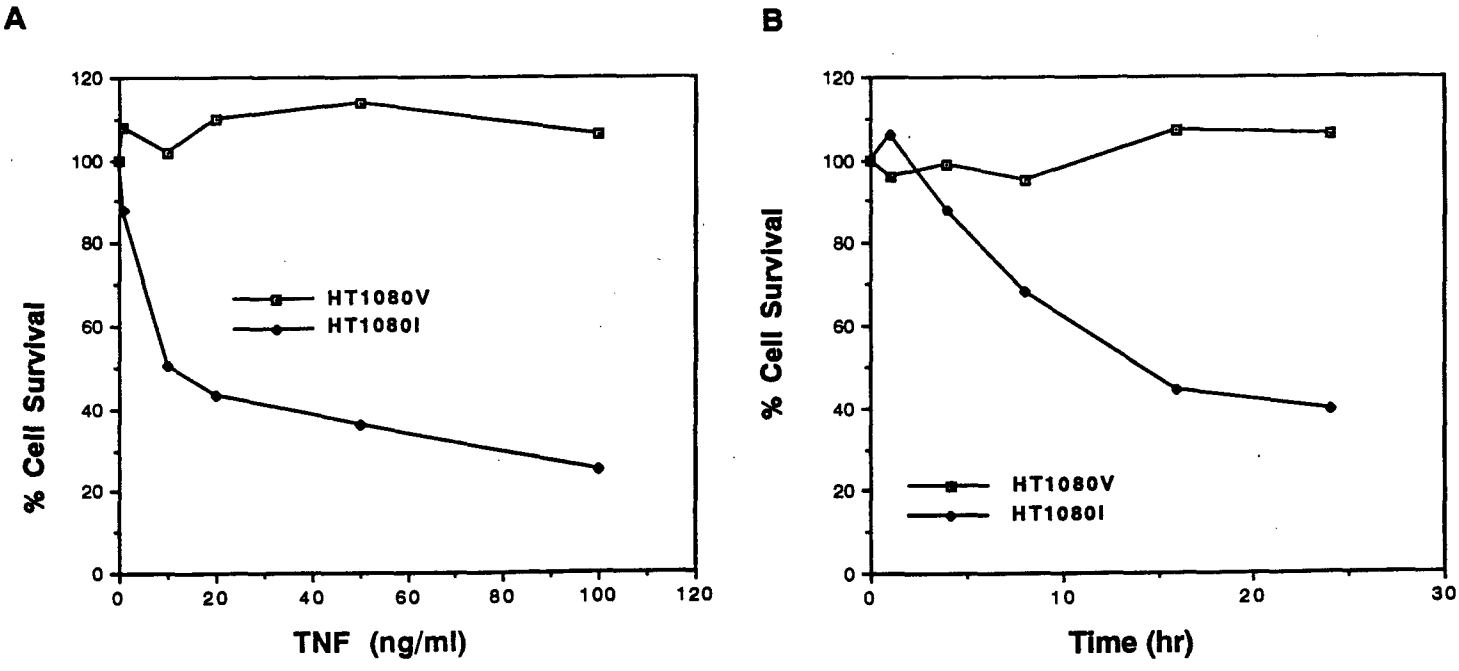

Figure 6. The expression of the super-repressor IkBa potently enhances apoptotic cell killing in response to TNF treatment. HT1080 cells (which are resistant to TNF-induced apoptosis) were transfected with the super-repressor IkBa and clones (HT1080I) were derived. A cloned carrying the vector was also derived (HT1080V). TNF treatment killed the HT1080I cell line with increased effectiveness as the concentration of TNF was increased (Figure 6A). The data is shown as % cell survival, comparing the vector control (V) versus the IkBa expressing cells (I). Figure 6B shows the % cell survival at different time points following TNF treatment and comparing the vector control versus the IkBa expressing cells (I). The data indicate that NF-kB protects against apoptotic cell killing induced by TNF.

Cancer Res., In press.

Internal Sequence Variations in the Ha-ras VNTR Rare and Common Alleles Identified by Minisatellite Variant Repeat Polymerase Chain Reaction (MVR-PCR)

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Running Title: Sequence variations in rare Ha-ras alleles

Key Words: Ha-ras, VNTR, minisatellite, polymorphism, polymerase chain reaction

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ABSTRACT

In this report, we describe the sequence allelotyping of the Ha-ras VNTR region using a minisatellite variant repeat (MVR)-polymerase chain reaction (PCR) approach. This method permits the rapid identification of internal sequence variations among the VNTR alleles, exploiting the presence of two polymorphic sites within the 28 bp repeat subunits which give rise to four distinct repeat types. Using MVR-PCR, 20 to 25 repeats at the 5' end of the VNTR can be sequenced rapidly and reliably. MVR typing of the common alleles a1, a2, a3 and a4 shows that the first 6 repeats at the 5' end of each allele constitutes an invariant region. Beginning with repeat 7, characteristic "signature" MVR patterns emerge for each common allele. The a1 and a2 common alleles were found to consist of specific repeat types 1, 2 and 3, while a3 and a4 contain an additional repeat type 4 not present in the smaller alleles. MVR typing of rare length alleles indicates they are comprised of disorganized sequences, although they usually bear a resemblance to one of the common alleles at the 5'-most end. These results suggest that the rare alleles may be generated from recombination or gene conversion-type events involving the common progenitor alleles. MVR typing could therefore improve the ascertainment of rare Ha-ras alleles and may provide molecular insights into the genesis of cancer-associated alleles.

INTRODUCTION

A number of carefully conducted studies strongly suggest that rare alleles of the Ha-ras1 protooncogene are associated with increased susceptibility to a variety of cancers (1-10). Although the mechanism underlying this association is unknown, the Ha-ras VNTR can function as an enhancer (11,12) and it has been suggested that the rare Ha-ras alleles may bind more avidly to transcription factors (13,14).

The polymorphism at the Ha-ras locus is due to the presence of a variable number tandem repeat (VNTR) located approximately 1 kb downstream from the protooncogene (15). Previous studies examining the association of rare Ha-ras alleles with cancer have utilized Southern blotting which is limited in its ability to adequately resolve small differences in allele lengths, especially for the larger alleles, and therefore may lead to allelic misclassification. In addition, the designation of alleles as common versus rare (1), or common, intermediate and rare (3,7) based simply upon population frequencies lacks a defining mechanism.

The subjectivity of Ha-ras allele length designations and the inaccuracies inherent in Southern hybridization methodology have led us to investigate other potentially more biologically-relevant structural features of the Ha-ras VNTR that may better correlate with cancer development. Studies by Jeffreys and coworkers (16-18) indicate that minisatellite alleles vary not only in repeat copy number but also in the interspersion pattern of repeat sequences along the VNTR. The Ha-ras VNTR sequence

derived from the EJ bladder carcinoma cell line (15) reveals G/C polymorphisms at positions 7 and 15 in the 28 bp repeat unit (Table 1). Additional point mutations also occur but these tend to cluster within the repeats located at the ends of the VNTR, consistent with findings in other minisatellite repeat loci (16).

In this study, we describe a modification of the minisatellite variant repeat (MVR) method originally described by Jeffreys et al (16) to identify sequence variations among Ha-ras VNTR alleles. Four repeat-specific primers corresponding to the two G/C polymorphic sites and a common anchored primer flanking the VNTR are used to PCR amplify fragments whose lengths define the positions of the repeat type along the VNTR. In effect, an allele-specific sequence polymorphism ladder is generated. Using a non-radioactive modification of MVR for rapid screening of DNA samples, we are able to reliably type at least 20 to 25 repeat units from the 5' end of the VNTR. This method provides a sequence-based differentiation of the rare from the common alleles.

MATERIALS AND METHODS

The MVR-PCR approach used is similar to the four-state MVR method described by Tamaki et al (19). An upstream primer (Amp L) 5'-GGTTGGGGGAGAGCTAGCAGGGCA-3' was synthesized from sequences flanking the 5' end of the VNTR. Four repeat-specific primers were synthesized complementary to the four possible repeat types within the VNTR. These repeat-specific primers span the region of each subunit harboring the G/C polymorphisms (Primer 1: 5'-

N(20)-GGCGTCCCCTGGAGAGAAGGGC-3', Primer 2: 5'-N(20)-GGCGTCCCCTG GAGAGAAGGGG-3',Primer 3:5'-N(20)-GGCGTCCCCTGGACAGAAGGGG-3',Primer 4:5'-N(20)-GGCGTCCCCTGGACAGAAGGGC-3' with N(20)=5'-TCCCGCGTCCAT GGCAGCTG-3'). The final 3' nucleotide for each of these repeat-specific primers was positioned at the first G/C polymorphism (Table 1). A 20 nucleotide TAG sequence was added to the 5' end of each repeat-specific primer. In addition, a primer specific for the TAG sequence (5'-TCCCGCGTCCATGGCAGCTG-3') was also synthesized. To prevent progressive shortening at each PCR cycle because of repeat-specific primers priming internally in PCR products, repeat type detection and subsequent amplification is uncoupled by adding a 20-nucleotide TAG sequence to each repeat-specifc primer and carrying out amplification with a low concentration of the repeat-specific primer and high concentrations of Amp L and TAG primers.

Amplification was carried out in four separate 40 ul reactions containing 570 ng genomic DNA, 1 μM Amp L, 1 μM TAG, 50 mM KCl, 45 mM Tris-HCl, pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.5 μM EDTA, 110 μg/ml bovine serum albumin, 2.5 units AmpliTaq polymerase (Perkin Elmer), 1 mM each deoxyribonucleotide triphosphate, and 10 nM of repeat-specific primer 1, 2, 3, or 4. Reactions were hot-started by adding the dNTPs after tubes reached 96°C. Amplification was carried out as a 2-step PCR with the following cycle parameters: 1 cycle of 96°C for 9 min followed by 27 cycles of 1.3 min at 96°C and 6 min at 70°C, with a final extension of 10 min at 70°C. The MVR-PCR product ladders were separated by electrophoresis in 4% Metaphor

agarose gels 40 cm in length using TBE buffer (45 mM Tris-borate, pH 8.3 and 2 mM EDTA) and were stained with ethidium bromide followed by destaining in water. Gels were photographed under ultraviolet illumination using a Polaroid MP-4+ camera system and type 667 film, and MVR codes beginning at the 5' end of the VNTR were read from the bottom of the gels.

RESULTS

We have adapted MVR technology to decipher sequence variations within the Ha-ras VNTR. In order to verify the specificity of our primers and to be certain that this method accurately identifies the VNTR internal sequence, we first compared the expected Ha-ras VNTR sequence with the MVR pattern obtained from the EJ bladder carcinoma cell line, for which the hemizygous VNTR sequence is known (15). The Ha-ras VNTR in the EJ line is an a1 length allele, and displays the MVR pattern 5'-001321-13113221311222... which corresponds to the A1a MVR subtype shown in Table 2. The predicted sequence, 5'-001321-14113221311223..., differs from the A1a pattern at positions 8 and 12. These changes of a type 4 to type 3 and a type 3 to type 2 repeats, respectively, may represent either errors in the reported sequence or mutations that occurred in our particular EJ clone. The fact that the EJ Ha-ras VNTR sequence corresponds exactly to the common A1a allele sequence, however, suggests the former possibility.

MVR-PCR of the Ha-ras VNTR distinguishes five types of repeats we refer to as 0, 1, 2, 3, and 4. Repeats 1, 2, 3 and 4 correspond to the four possible sequence combinations resulting from the two G/C polymorphisms within the 28 bp subunits (Table 1). The 0 or null repeats, which are seen as blank positions on the gels, contain mutations which prevent primer binding and are therefore unamplifiable. Between 20 and 25 repeats could be reliably typed from the 5' end of the VNTR using this technique.

Using MVR, we proceeded to define the range of sequence allelotypes for the four common length Ha-ras VNTR alleles. All length assignments were previously derived using Southern hybridization methodology (7). Since the a1 common allele is present at the highest frequency in the population and since homozygous MVR patterns are the simplest to interpret displaying only a single band at each ladder position, we began the analyses on individuals known to be homozygous for the a1 allele. A total of 40 a1/a1 germline DNA samples were evaluated (Table 3). Seventy-eight of 80 a1 alleles showed one of two common MVR patterns identified as A1a or A1b. These two A1 MVR subtypes differ only at positions 17 and 18 as shown in Table 2 and Figure 1.

Once the a1 pattern was firmly established, we performed MVR typing on a1 heterozygotes (a1/a2, a1/a3, a1/a4) in order to deduce the patterns of the other common alleles. These sequences were determined by subtracting out the A1a or A1b pattern from the dual pattern seen in these individuals. This approach was previously used by Jeffreys et al (16) in their pedigree analysis

of the MS32 VNTR. The putative a2, a3 and a4 MVR patterns were then verified in homozygotes, although these allelotypes occurred very infrequently (Figure 1).

The MVR sequences (repeats 1-20) for the common length alleles are given in Table 2. The MVR pattern (5'-001321...3') over the first 6 repeats at the 5' end of all alleles was exactly the same. Because the first two repeats are 0 repeats, the first readable repeat is repeat position 3, seen as a 156 bp fragment at the bottom of the gels (Figure 1). Beginning with repeat position 7, each common length allele displays a characteristic "signature" sequence. One MVR sequence was identified for each of the common alleles a2 and a4, while 2 MVR subtypes were identified for a1 (A1a and A1b) and 3 were found for a3 (A3a, A3b and A3c), each differing by only one or two repeats from the other at the positions indicated in Table 2. Of all a1-length alleles analyzed, 97.5% had the exact A1a (5'-001321-13113221311222...)(77.4%) or A1b (5'-001321-13113221312122...)(20.1%) sequence over the first 20 repeats (Table 3). Of a2-length alleles, 90% displayed the exact A2 MVR sequence (5'-001321-22221311313123). For a3-length alleles, 92.3% were of the A3a (5'-001321-21132141224123...)(30.8%), A3b (5'-001321-21132111241231...)(38.5%) or A3c (5'-001321-21132141324123...)(23.1%) subtypes, while 97.5% of a4-length alleles had the A4 sequence (5'-001321-41224123241222...). Interestingly, the a1 and a2 alleles are composed solely of 3 specific 28 bp repeat types (types 1, 2 and 3)(Table 2), while a3

and a4 are characterized by the presence of the type 4 repeat not found in a1 and a2.

The remainder of the common length alleles were minor variants (denoted by -V) of the MVR sequence subtypes described above. These variants, shown in Table 4, were characterized as having one or two repeat alterations occurring as additions, deletions or changes in repeat types in otherwise common MVR sequences. The frequencies of variation from the established MVR patterns were 2.5% for a1-length alleles, 10% for a2 alleles, 7.7% for a3 alleles and 2.5% for a4 alleles (Table 3). All of the MVR structural variants identified involved intact 28 bp subunits. We did not detect any Ha-ras VNTR alleles containing repeat units of abnormal length (either shorter or longer than the expected 28 bp) which would place the MVR ladders of the two constituent alleles out of register.

In contrast to the common length alleles which have characteristic and relatively stable sequences, MVR typing of six germline DNA samples previously shown to have rare length alleles by Southern analysis indicated that these alleles possessed disorganized internal sequences. The sequence alterations involved more than two repeats, and for some rare alleles, involved the entire 5' sequence. The rare alleles R343, R45, R348 and R397, shown in Table 2, appear to be derived at the 5' end from the common alleles A1, A3 or A4. The R348 rare allele (5'-001321-21132121132121...) possesses a sequence identical to A3 through repeat 12. However, beginning with repeat 13, the A3 pattern is disrupted. Similarly, the R45 (5'-001321-

41222221311124...) and R397 (5'-001321-41222221311124...) rare alleles begin as A4 alleles but again the A4 sequences are lost farther downstream. Moreover, the R37 (5'-001321-11113311311311...) and R379 (5'-001321-23113214232412...) rare alleles bear little resemblance to the common alleles.

This MVR method permits us to read the sequence of approximately 25 repeats from the 5' end of the VNTR, so that nearly the complete MVR sequence of the a1 common allele can be characterized. However, only about 35% of the a4 common allele can be determined. Despite this inherent bias toward sequence ascertainment of the smaller alleles, we were still able to uncover significant sequence variation for large alleles known to have rare lengths.

DISCUSSION

A number of recent studies have focused on the putative role of Ha-ras rare alleles as markers of genetic susceptibility to a variety of cancers, including cancers of the breast, lung, and bladder(1-10). In this report, we have described an adaptation of the MVR technique for sequence allelotyping of the Ha-ras VNTR. Our preliminary analyses of the 5' end of the VNTR indicate that Ha-ras allele length is closely linked with allele sequence for the common alleles, a1, a2, a3 and a4, in that each common length allele possesses its own characteristic sequence(s). For example, nearly all a2 length alleles possess the same identical 5' MVR sequence. In contrast, MVR typing of six rare length alleles suggests that these alleles are composed

of disorganized internal sequences, although they appear to be derived in most cases from common progenitor alleles. A previous report by Kasperczyk et al (20) suggested that rare alleles are derived from the common allele nearest in size. Based upon MVR sequence typing, this does not appear to be true for the rare allele R45. The R45 allele lies between a2 and a3 in length, yet possesses an a4-like MVR sequence at the 5' end. Thus, this rare allele appears to be derived from an a4 allele rather than a2 or a3.

Jeffreys and coworkers have extensively characterized a number of other VNTRs, including MS32 (16, 19), MS205 (17) and MS31A (18), using the MVR technique. These VNTRs, which have been used for DNA fingerprinting, have polymorphic frequencies of 95% or more based on length differences alone, and by MVR exhibit high levels of allelic variation. In contrast, the Ha-ras VNTR has a much lower level of population heterozygosity (approximately 65% by length as determined by Southern hybridization)(21) and the common Ha-ras alleles appear to be relatively stable, maintaining a strict association between length and sequence.

The rare Ha-ras VNTR alleles, however, appear to have resulted from recombinatory events as evidenced by the resemblance to common allele MVR patterns at the 5' end of the VNTR but with significant rearrangement of the internal repeats. Jeffreys has suggested that inter-allelic recombination or gene conversion-like events are responsible for the allelic variation at the MS32 and MS31A loci (22) as evidenced by the preservation

of VNTR flanking regions but rearrangement of internal sequences. Presumably, this rearrangement could occur via meiotic recombination since the VNTR alteration is present in the germline.

These data raise the possibility that rare alleles are a reflection of an inherent genetic instability which is the direct effector of cancer susceptibility. MVR therefore can be utilized for the rapid and precise identification of the rare Ha-ras variants within a population. In addition, the analysis of repeat architecture at the Ha-ras locus may provide clues to the mechanism of cancer association with the rare alleles.

ACKNOWLEDGEMENTS

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Table 1: Sequence Polymorphisms within the 28 bp Subunits of the Ha-ras VNTR

MVR Repeat Type	Repeat Sequence	No. Repeats in EJ cell line
[a] 1:	5'-a c a c t c **G** c c c t t c t **C** t c c a g g g g a c g c c-3'	12/29
2:	.....**C**.....**C**.....	7/29
3:	.....**C**.....**G**.....	5/29
4:	.....**G**.....**G**.....	1/29

Polymorphisms are shown at positions 7 and 15 from the 5' end. [a]Sequence 1 is the 28 bp consensus sequence present in 12 of the 29 repeats in the EJ VNTR.

Table 2: MVR Patterns of Common or Rare Length Ha-ras VNTR Alleles

		COMMON ALLELES							RARE ALLELES											
	Repeat	A1a	A1b	A2	A3a	A3b	A3c	A4	R343		R37		R45		R379		R348		R397	
	Position	1.00 kb[a]		1.45		2.05		2.5	1.175		1.225		1.750		1.750		2.225		2.225	
		(0.78)[b]	(0.20)	(1.00)	(0.31)	(0.35)	(0.23)	(1.00)												
5'	1	0	0	0	0	0	0	0	0		0		0		0		0		0	
	2	0	0	0	0	0	0	0	0		0		0		0		0		0	
	3	1	1	1	1	1	1	1	1		1		1		1		1		1	
	4	3	3	3	3	3	3	3	3		3		3		3		3		3	
	5	2	2	2	2	2	2	2	2		2		2		2		2		2	
	6	1	1	1	1	1	1	1	1		1		1		1		1		1	
	7	1	1	2	2	2	2	4	**1**	(A1)[d]	1	(?)	**4**	(A4)	2	(?)	**2**	(A3)	**4**	(A4)
	8	3	3	2	1	1	1	1	**3**		1		**1**		3		**1**		**1**	
	9	1	1	2	1	1	1	2	**1**		1		**2**		1		**1**		**2**	
	10	1	1	2	3	3	3	2	**1**		1		**2**		1		**3**		**2**	
	11	3	3	1	2	2	2	4	**3**		3		**4**		3		**2**		2	
	12	2	2	3	1	1	1	1	**2**		3		**1**		2		**1**		2	
	13	2	2	1	(4)	(1)	(4)	2	**2**		1		**2**		1		2		2	
	14	1	1	1	1	1	1	3	**1**		1		**3**		4		1		1	
	15	3	3	3	(2)	(2)	(3)	2	2		3		1		2		1		3	
	16	1	1	1	2	4	2	4	2		1		3		3		3		1	
	17	(1)[c]	(2)	3	4	1	4	1	3		1		3		2		2		1	
	18	(2)	(1)	1	1	2	1	2	1		3		4		4		1		1	
	19	2	2	2	2	3	2	2	3		1		1		1		2		2	
3'	20	2	2	3	3	1	3	2	1		1		2		2		1		4	

A; common allele, R; rare allele, a; allele length in kilobase pairs determined by Southern hybridization, b; proportion of each common allele with this MVR pattern, c; variant repeat positions in a1 or a3 alleles are indicated by ( ), d; bolded repeats in the rare alleles R343, R45, R348 and R397 indicate 5' sequence similarity to the common alleles A1, A3 or A4 as indicated.

Table 3: MVR Sequence Variation in the Common Length Ha-ras Alleles a1, a2, a3 and a4

Length [a]Allelotype	No.Alleles Analyzed (No. Persons)	NUMBER of COMMON LENGTH ALLELES with this MVR SEQUENCE													
		a1 Alleles				a2 Alleles		a3 Alleles						a4 Alleles	
		A1a	A1a-V[b]	A1b	A1b-V	A2	A2-V	A3a	A3a-V	A3b	A3b-V	A3c	A3c-V	A4	A4-V
a1/a1	80 (40)	60	2	18	0	-	-	-	-	-	-	-	-	-	-
a1/a2	80 (40)	36	0	4	0	36	4	-	-	-	-	-	-	-	-
a1/a3	78 (39)	29	1	8	1	-	-	12	1	14	2	9	0	-	-
a1/a4	80 (40)	29	1	10	0	-	-	-	-	-	-	-	-	39	1
Total Alleles	328 (164)	154	4	40	1	36	4	12	1	14	2	9	0	39	1
Sequence Variants in the Common Length Alleles (%)		5/199 (2.5)				4/40 (10.0)		3/39 (7.7)						1/40 (2.5)	

[a]Allele length determined by Southern blotting methods
[b]-V; sequence variants displaying one or two repeat differences within the first 20 repeats at the 5' end as compared with the known common sequence(s)
--; none found

Table 4: Variants of the Ha-ras VNTR Common Allele MVR Sequences

	Repeat Position	A1a-Var			A1b-Var	A2-Var				A3a-Var	A3b-Var		A4-Var
		V75	V178	V214	V525	V128	V139	V270	V283	V325	V248	V135	V581	V33
5'	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	1	0	0	0	0	0	0	0
	3	1	1	1	1	1	3	1	1	1	1	1	1	1
	4	3	3	3	3	3	2	3	3	3	3	3	3	3
	5	2	2	2	2	2	1	2	2	2	2	2	2	2
	6	1	1	1	1	1	2	1	1	1	1	1	1	1
	7	1	1	1	1	1	2	2	2	(1)	2	2	2	4
	8	(2)	3	3	3	3	2	2	2	2	1	1	1	1
	9	3	1	1	1	1	2	2	2	2	1	1	1	2
	10	1	1	1	1	1	1	2	2	2	3	3	3	2
	11	1	2	3	3	3	3	1	1	1	2	2	2	4
	12	3	2	2	2	2	(3)	3	3	3	1	1	1	1
	13	2	1	2	2	2	1	1	1	1	1	1	1	2
	14	2	3	(2)	1	1	1	1	1	1	2	(2)	1	3
	15	1	1	1	3	3	3	3	3	3	2	2	2	2
	16	3	1	3	1	1	1	1	1	1	4	4	4	4
	17	1	2	1	1	2	3	3	3	3	1	1	1	1
	18	1	2	1	2	1	1	1	1	1	2	2	(1)	2
	19	2	2	2	2	2	2	2	(1)	2	3	3	3	2
3'	20	2	3	2	(3)	(3)	3	(2)	2	3	2	1	1	(1)

[a]; variant repeat positions in the common MVR sequences are indicated by ( )for a change in repeat type or insertion of a repeat, or _ for deletion of the following repeat.

LEGENDS TO FIGURES

Figure 1: Examples of Ha-ras VNTR MVR patterns from individuals with common or rare alleles.

MVR-PCR was performed using primers specific for the four repeat types corresponding to the G/C polymorphisms identified from the EJ bladder carcinoma VNTR sequence. (A) MVR patterns of individuals homozygous for the common length alleles a1, a2 or a4, (B) MVR patterns of heterozygous individuals with a1/a2 or a2/a4 common length alleles, (C) MVR patterns of heterozygous subjects with one common and one rare length allele. The heterozygous MVR allelotypes shown are A1a/R37, A2/343, A4/348, and A4/379. The first readable repeat for each subject is the 156 bp fragment corresponding to repeat 3 at the bottom of each gel.

Figure 1a

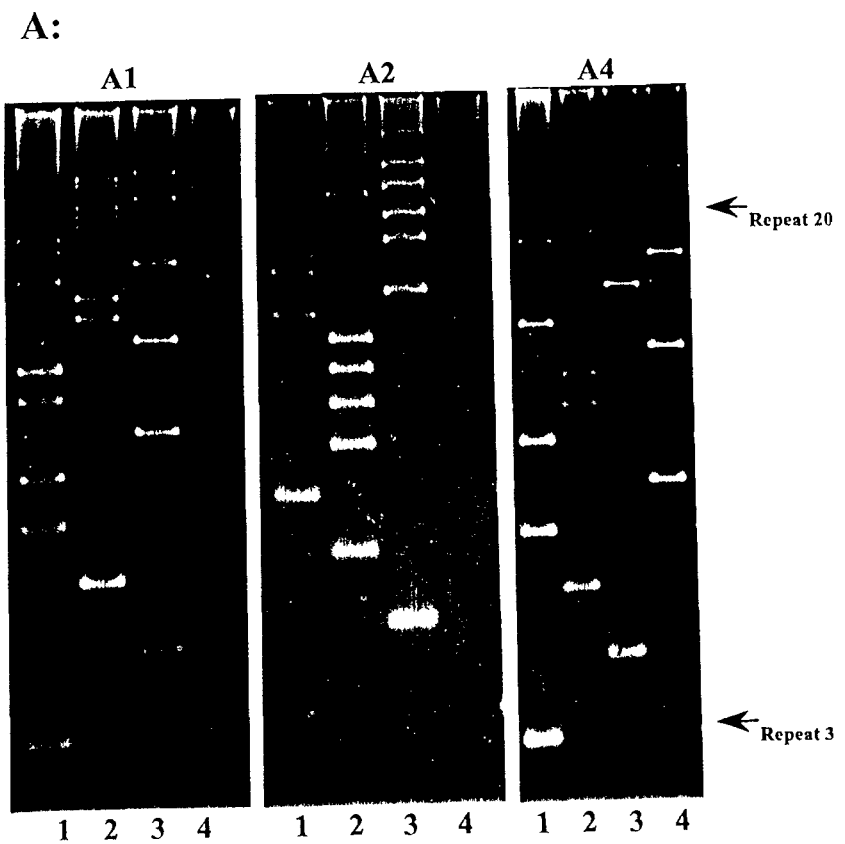


Figure 1b

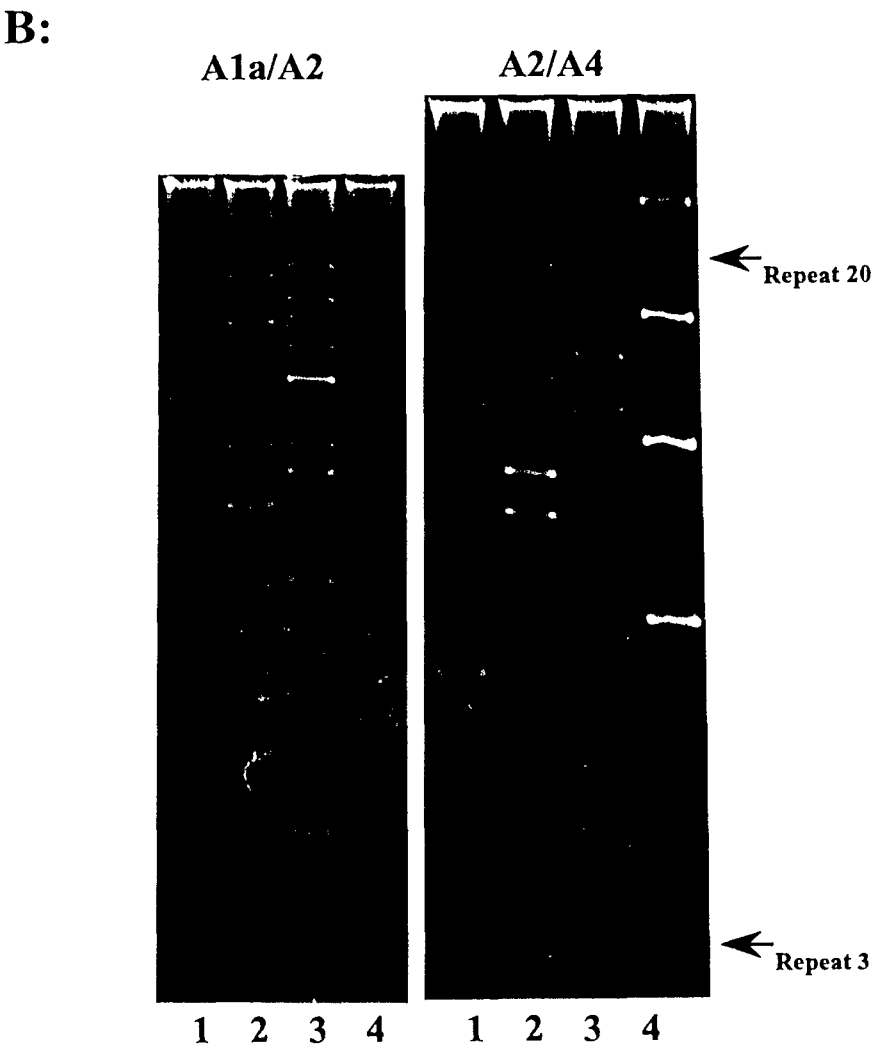
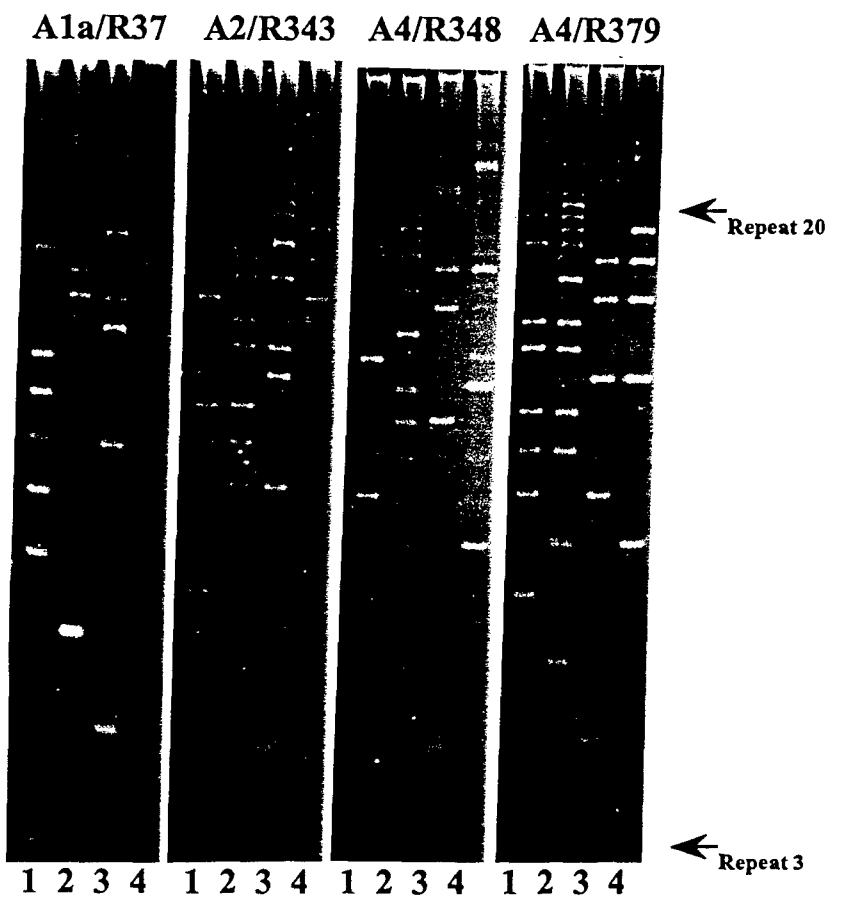


Figure 1c

C:


A1a/R37
A2/R343
A4/R348
A4/R379
Repeat 20
Repeat 3
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

DEPARTMENT OF THE ARMY
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REPLY TO
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